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MOLECULAR DETECTION OF POTATO LEAF ROLL POLEROVIRUS THROUGH REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION IN DORMANT POTATO TUBERS

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Abstract

A Reverse Transcription-Polymerase Chain Reaction (RT-PCR) technique was applied for the detection of *Potato leaf roll polerovirus* (PLRV) in dormant potato tubers. A primer pair was designed from the coat protein-encoding fragment of the PLRV genome that amplified a 336-bp product. The amplified product was detected in nucleic acid preparations from leaves and tubers of 5 cultivars and from purified virions. The specificity of the RT-PCR product was confirmed through southern blot analysis. The primer pair used in the RT-PCR did not produce any non-specific product from 7 other potato viruses. Sensitivity of RT-PCR was confirmed by detecting PLRV from known mixture of PLRV and randomly selected potato virus. Dilution of 1:1000–1:4000 and 1:200–1:1000 were used to detect viral load from foliage and tuber, respectively. RT-PCR efficiently detected PLRV in sprouting tubers as well as dormant tubers stored at 20– 25°C for 4 months.

Introduction

Potato leaf Roll Virus (PLRV) is the most economically important virus infecting potato (*Solanum tuberosum* L). PLRV is distributed world wide in potato growing areas. Quality of seed tubers is badly affected due to the development of phloem necrosis. The diseased plants produce fewer and smaller tubers than the normal plants resulting in significant yield reduction.

PLRV is among the important potato viruses in Pakistan and is widely distributed in the country, with an incidence of 15-65% (Mughal *et al*., 1988). Mirza (1978) reported that aphid (*Myzus persicae*) is responsible for rapid spread of PLRV in the spring potato crop in the Punjab. Recent surveys have confirmed that PLRV is among the most prevalent and economically important viruses of potato in Pakistan (Jan & Khan, 1995; Ahmed & Ahmed, 1995).

PLRV is the type member of the genus Polerovirus and belongs to the family Luteoviridae. It has a monopartite, non-polyadenylated RNA genome of ~6 KB. Some basic properties of a field isolate of PLRV in Pakistan were studied by Arif *et al*., (1995). The successful elimination of potato viruses such as PLRV from seed potato through tissue culture (Awan *et al*., 2007) requires a specific and sensitive method of virus detection. Serological methods can be unreliable for the detection of PLRV, this virus often occurs at low concentration in plant tissue and virions are weakly immunogenic (Beemster *et al*., 1987). PLRV has not been reliably detected from the primarily infected tubers either in the dormant stage or after the natural break of dormancy. Break down of dormancy is required to detect PLRV in dormant potato tubers through ELISA (Vetten *et*

al., 1983). Reverse Transcription Polymerase Chain Reaction (RT-PCR) offers a potentially more sensitive method for detection of PLRV and other viruses in dormant tubers. This study was conducted to develop an RT-PCR protocol for detection of PLRV in dormant tubers of cultivars grown in Pakistan.

Materials and Methods

Virus cultures and inoculation: PLRV-infected potato tubers were collected from Jassoky Farms, Okara, Pakistan during January 2003. The tubers were planted in the field to obtain material for *in vitro* isolation. After sprouting, plantlets were tested by double antibody sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) to confirm the occurrence of single infection of PLRV. Infected tissues was harvested, preserved and used as positive control. Plants infected by PLRV were detected by DAS-ELISA according to the supplier's protocol (BioReba). The reaction was stopped by adding 70µL of 3M NaOH, and absorbance measured at 405 nm in a microplate reader (BioTek, USA). Virus-free plantlets of potato cvs., Courage, Desiree, Diamant, Cardinal and Sante were grown in a greenhouse. Inoculum from infected leaves was prepared in a buffer solution (0.01 M Sodium phosphate containing 0.4% Sodium sulfite, pH 7.5). To optimize PLRV infection of tubers, potato plants were top grafted with PLRV-infected scions. Plants were assayed by DAS-ELISA, 30 and 60 days post-grafting, and tubers were harvested 90 days post-grafting and tested within 2–3 weeks (dormant), followed by testing at 2 and 4 months after storage at 20–25°C. Potato viruses A (PVA), M (PVM), S (PVS), X (PVX) and PVY strains and potato spindle tuber viroid (PSTVd) from virus collection were used to test the specificity of the PLRV primers. To determine whether PLRV can be specifically detected in the presence of PVY, leaves from Cardinal plants infected with different isolates of PVY, and mixed with PLRV-infected leaves of Diamont plants, were tested by RT-PCR for PLRV detection as described below.

RNA extraction from potato tissues and tubers: RNA extraction was conducted using a kit according to available protocol. Briefly, potato tubers were peeled cut into small pieces and ground, transferred into centrifuge tubes and centrifuged at 14,000 rpm for 15 min. in a bench-top micro centrifuge (Eppendorf Co., USA). An aliquot of 150 µl potato sap was taken and mixed with 100 µl Gentra Cell lyses solution. After incubation at room temperature for 1-2 minutes, 100µl of DNA protein binding Reagent was added, mixed well and incubated on ice for 5-10 minutes. Extracts were centrifuged for 10 minutes at 14,000 rpm. Clear supernatant was pipetted into a tube containing chilled 500µl isopropanol and mixed thoroughly. Supernatant was discarded after centrifugation for 10 minutes at 14,000 rpm. The pellet was washed with 70% ethanol (1ml) by gently inverting tube 5-6 times, air dried and re-suspended in 30-50µl DEPC (Diethylpyrocarbonate) treated water and immediately stored at –70º C for future use., Fresh leaves were extracted as above after grinding in liquid nitrogen with a mortar and pestle. For the composite samples, tuber sap containing PLRV was mixed with sap from non-infected tubers in a ratio of 1:4, 1:9, 1:14, 1:19, 1:29 and 1:39 prior to nucleic acid extraction.

Reverse transcription: RT-PCR was performed using primers designed for Capsid protein gene of PLRV. cDNA was synthesized by denaturing 5µl RNA solution, containing 1mM dNTPs, 10pmol antisense primer for (PVY) and water, for 5 minutes at

65°C. Samples were immediately placed on ice and add to it 5X 1st strand buffer, DDT & 1 unit of (M-MLV). RT mix was subjected to two thermo-cycling conditions at 37°C, 50 minutes and 70°C, 15 minutes.

Polymerase chain reaction:A total volume of 20µl, containing 10X PCR buffer (1.2mM $MgCl_2$, 10mM tris-HCl pH 8.0), 1mM of each deoxyribonucleoside triphosphate (dNTP), 10 pmoles of each forward (5'GCAATGGGGGTCCAACTCAT 3') and reverse (5'CGCGCTAACAGAGTTCAGCC3') primer (primer were designed using coat protein gene sequence; accession no. DQ269981.1), 1 unit Taq DNA polymerase and cDNA template, was used. It was overlaid by 20µl light mineral oil on the reaction mixture. To amplify DNA fragments of 336 bp from the cDNA of PLRV, PCR conditions for PLRV was as follows: one cycle of 5 min at 94°C and 35 cycles (45 sec at 94°C, 45 sec at 58°C, 45 sec at 72°C, followed by 10 min final extension at 72°C in a thermocycler. A PCR mix without template was used as negative control. Annealing temperature for primers was optimized at 58°C after setting up PCR at various annealing temperatures. PCR products were separated by electrophoresis on a 1% agarose gel together with molecular weight marker (Fermentas), stained with ethidium bromide and visualised under UV light. MJ Research PTC-100 thermocycler (MJ Research International, USA) was used for all polymerase reactions.

Southern blot analysis: RT-PCR product was resolved on 1% agarose and then transferred onto Hybond-N membrane (Amersham, USA). Hybridisation was carried out using the gene specific labelled probes (*Sambrook et al*., 1989).

Results

Forward and reverse primers were designed from the coat protein gene sequence of the PLRV genome. 336 bp DNA fragment was amplified through Reverse Transcription Polymerase Reaction from virus RNA isolated from PLRV infected leaves (lane 1), tubers (lane 2), and purified virions (lane 3). No DNA band was visible in PCR amplification from plant tissues not infected with PLRV (Fig. 1. lane 4-5).

Specificity of RT-PCR product was demonstrated through Southern Blot analysis. A DNA specific probe gave sharp bands on nitrocellulose membrane at expected size of 336 bp (Fig. 2.). Total RNA preparations from Potato viruses PVA, PVM, PVS, PVX, PVY^{O}, PVY^{N}, PVY^{NTN}, and PSTVd were used as template for RT-PCR. Agarose gel did not show any amplified product. RNA extracts from leaves and tubers diluted up to 1:4000, prior to the RT-PCR amplification, showed that PLRV was reliably detected in leaves up to 1:1000 dilution and faintly up to 1:4000 from leaf extracts and in tubers up to 1:200 and in some samples up to 1:1000 (Fig. 3).

Specificity of RT-PCR was studied by detecting PLRV in mixed samples with the common co-infection i.e potyvirus, PVY, as shown (Fig. 4, Lanes 2-8), a 336-bp product amplified specific to PLRV. There was no signal of non-specific DNA band for PLRV indicative of cross-amplification, and no bands were obtained with uninfected samples (Fig. 4 Lanes 9-12).

Primarily infected tubers of 5 cultivars viz., Courage, Desiree, Diamant, Cardinal and Sante were tested in the dormant stage as well as after 2 and 4 months of storage. Detection of PLRV infection in 100% of infected tubers confirmed the high reproducibility of results of RT-PCR detection system. Storage of tubers at 20–25°C for 2 and 4 months did not affect the detection limit of PLRV.

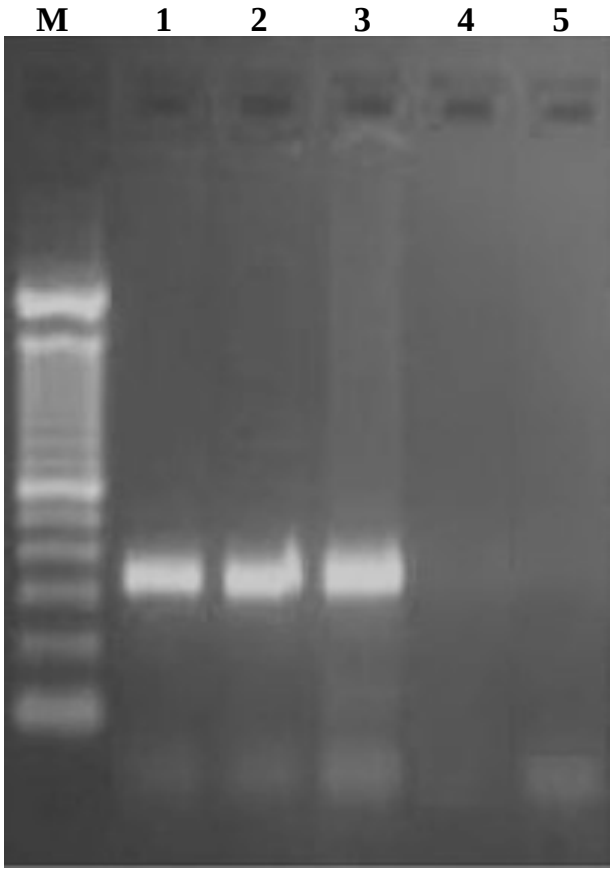

Fig. 1. Lane M= 100 bp Ladder: Lane 1= PCR amplified fragments of PLRV from nucleic acid preparations of infected leaves, Lane 2 from nucleic acid preparations of tubers and Lane 3 from nucleic acid preparations of purified virions. Lane 4-5= non-infected tissues.

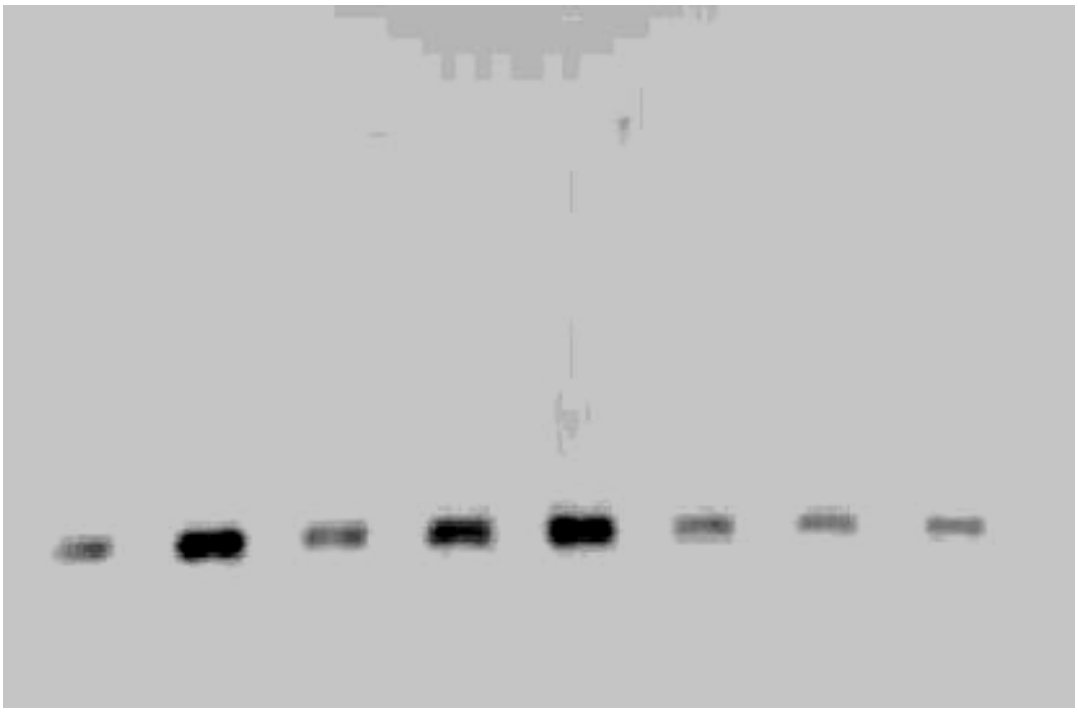

Fig. 2. Southern blot hybridization with digoxigeninlabeled PLRV- cDNA probe of reverse transcription polymerase chain reaction products of PLRV-infected samples.

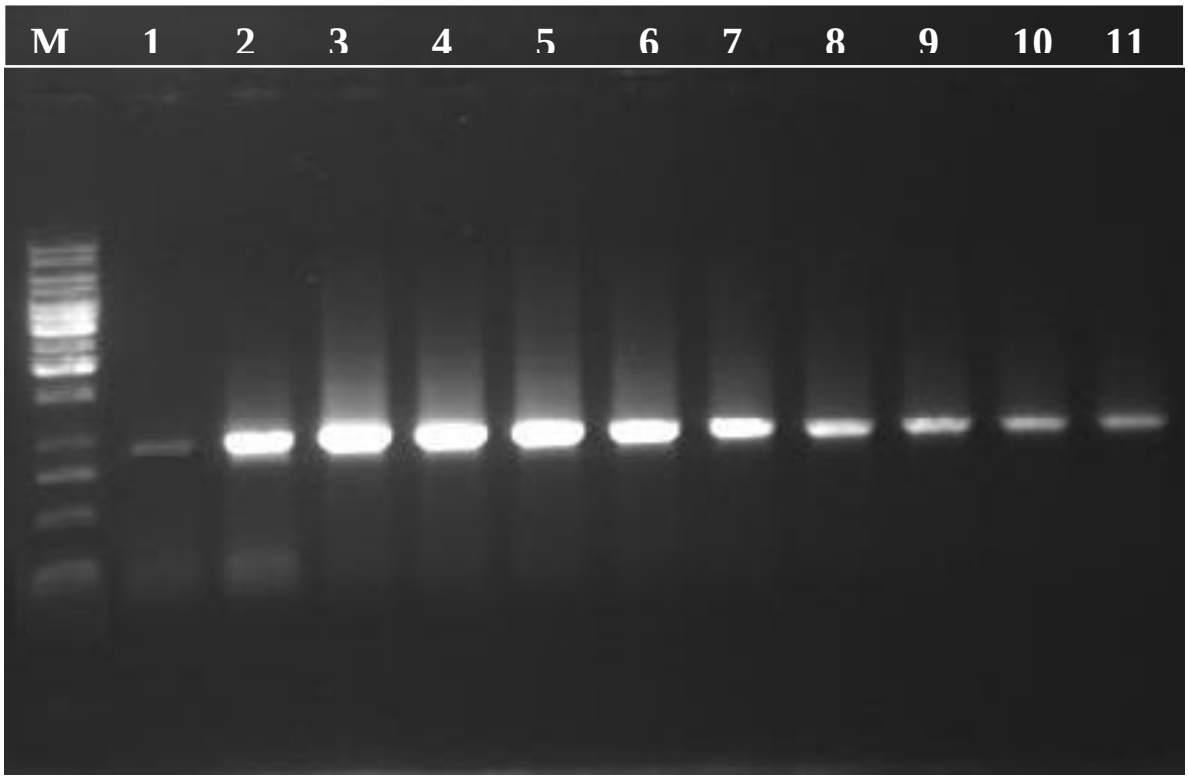

Fig. 3. Sensitivity of PLRV detection by reverse transcription polymerase chain reaction in leaves (Lanes= 2-6) and in tubers (Lanes=7-11), M=Size Marker, Lane1= Positive control.

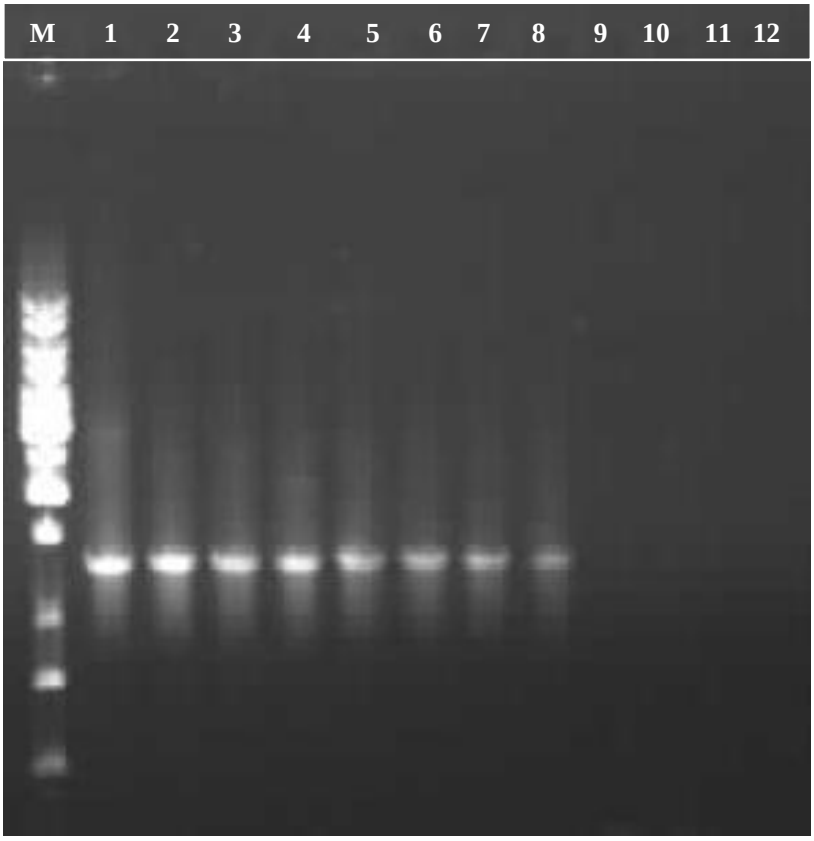

Fig. 4. Specific detection of 336-bp fragment PLRV in mixed samples with co-infection of Potato virus Y (PVY). Lane 1= Positive Control (PLRV only), Lanes 2-8= sap containing PLRV and PVY, Lanes 9-12= non-infected samples.

Discussion

This study emphasized a reliable molecular detection protocol based on RT-PCR for testing the presence of PLRV in dormant potato tubers in particular and the foliage in general, involving the small sample size. The PCR provides good alternative to other diagnostic methods having not only high sensitivity but also often eliminates the need for radioactive probes. Hadidi & Yang (1990) reported the detection of viriods in apple scar skin group from total nucleic acid extracts of infected pome fruit trees by RT-PCR amplification. In their experiments RT-PCR has been providing the flexibility to amplify any segment of a genome by selecting a pair of primers from the segment. The primer pair used in this study amplified a 336 bp product using coat protein genomic fragment of PLRV as template. Southern Blot precisely proved the précised specificity of amplified product. RT-PCR for Nucleic acids preparations from PVA, PVM, PVS, PVX, PVY^{O}, PVY^{N}, PVY^{NTN}, and PSTVd showed no amplification and revealed the capacity of these primers to anneal with high precision to detect the PLRV in mixed viral infection. Hadidi *et al.* (1993) showed RT-PCR detection of potato leaf roll luteovirus (PLRV) from infected potato leaves and viruliferous aphids. In this study we demonstrated the efficient molecular detection of PLRV from dormant tubers stored at 20-25°C for four months. Results show that on the basis of the indicator plants and ELISA, concentration of PLRV in potato leaves and tubers is considerably lower than other potato viruses. In the case of PVY, tuber extracts diluted to 1:4000 could provide enough nucleic acid templates for effective RT-PCR (Singh & Singh, 1996; Singh & Singh, 1997). However, PLRV was not detected beyond 1:1000 dilution in the tuber extracts of infected cultivars. As noted in PVY infected plants (Singh & Singh, 1995), the concentration of PLRV is also lower in the tubers than in the leaves. Different molecular methods were developed by Singh (1998) for the detection and prevention of different potato viruses and viroids. It is considered that small size of amplicon can increase PCR sensitivity (Singh, 1998). In this study, the size of the PCR product was also limited to 400 bp to increase the specificity of the test, and this size range provided the same level of sensitivity. In some studies, Real Time PCR has been described for efficient detection of PLRV in dormant tubers (Mortimer-Jones *et al*., 2009; Agindotan *et al*., 2007) but due to high costs of reagents and equipment involved in real time PCR, the method described herein is more applicable and cost-effective.

We demonstrated a reliable molecular detection system based on RT-PCR, which was optimized on locally grown cultivars. The system can be applied for preliminary diagnosis of viral load in potato tubers, improve the quality of seed potato and ultimately enhance productivity of potato crop.

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